Analysis of the mutant *proBA* operon from a proline-producing strain of *Serratia marcescens*

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The nucleotide sequence of the *proBA* operon from a proline-hyperproducing mutant of *Serratia marcescens* was determined. Two base substitutions were found: one in the *proB* structural gene, coding for γ -glutamyl kinase (GK), and a second one in the promoter region of the operon. The former base substitution led to a change of the predicted amino acid at position 117 from an alanine to a valine in GK. This mutation rendered GK 700-fold less sensitive to proline-mediated feedback inhibition than the wild-type enzyme. The other base substitution, a transversion from a G–C to an A–T, was located in the spacer region between the '–35' and '–10' sequences of the promoter, and it increased the transcriptional activity of this operon fourfold. Both these two base substitutions, which were acquired at the step of selecting mutants resistant to a toxic proline analogue, 3,4-dehydropoline, confer upon cells a high proline productivity and an increased osmotolerance.

Introduction

Proline is one of the important cellular osmolytes that are closely related to resistance to osmotic stress. The accumulation of proline decreases intracellular water potential and, therefore, makes various organisms osmotolerant (Aspinall & Paleg, 1981; Measures, 1975). Proline accumulation can be a result of proline uptake from the medium or increased proline biosynthesis.

In micro-organisms, proline is synthesized from glutamate via three enzymic reactions, catalysed by γ -glutamyl kinase (GK; *proB* product), γ -glutamyl phosphate reductase (GPR; *proA* product), and L-pyrroline-5-carboxylate reductase (*proC* product). We have been working on proline metabolism in *Serratia marcescens*. Wild-type strains of this bacterium do not grow in minimal medium with increased osmolarity and produce only a small amount of proline (Sugiura & Kisumi, 1985a; Sugiura *et al.*, 1985). Proline-analogue-resistant

mutants have been isolated from *S. marcescens* and tested for proline productivity and osmotic stress tolerance (Sugiura & Kisumi, 1985a, b; Sugiura *et al.*, 1985). Some of these mutants showed enhanced proline productivity and enhanced osmotolerance. One of them, SP187, produced more than 60 g of L-proline l⁻¹ in a fermentation medium (Sugiura & Kisumi, 1985a).

We have recently determined the nucleotide sequence and the gene structure of the *proBA* operon from wild-type *S. marcescens* (Omori *et al.*, 1991). *S. marcescens* is similar to *Escherichia coli* in the gene structure and the amino acid sequences of the two enzymes encoded by this operon. Proline synthesis from glutamate is regulated mainly through proline-mediated feedback inhibition of GK, the first enzyme in the pathway.

Strain SP187 described above was obtained by four rounds of mutagenesis and selection. This strain has the *putA* mutation, abolishing proline oxidase activity, and shows resistance to the proline analogues 3,4-DL-dehydropoline (DHP), L-thiazolidine-4-carboxylic acid (TAC) and L-azetidine-2-carboxylic acid (Sugiura & Kisumi, 1985a). Transductional analysis suggested that the DHP resistance mutation, designated the *dpr-1* genotype, might be closely linked to the *proBA* region but we had no further information on this mutation (Sugiura & Kisumi, 1985a).

In the work described here, we determined and analysed the nucleotide sequence of the mutant *proBA*

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Abbreviations: GK, γ -glutamyl kinase; GPR, γ -glutamyl phosphate reductase; DHP, 3,4-DL-dehydropoline; TAC, L-thiazolidine-4-carboxylic acid.

The nucleotide sequence data reported in this paper have been submitted to GenBank, EMBL and DDBJ, and have been assigned the accession number D90351.

operon, which was previously cloned from strain SP187 (Takagi *et al.*, 1985).

Methods

Strains, plasmids and media. The strains and plasmids are listed in Table 1. *E. coli* JM109 was used for the construction of plasmids. The rich medium used was LB (Davis *et al.*, 1980); the minimal medium of Davis & Mingioli (1950) was modified by omitting the sodium citrate and supplemented with 0.5% glucose or 0.5% disodium succinate hexahydrate as a carbon source. Required L-amino acids were used at 1 mM. Antibiotics were added at the following concentrations: kanamycin (Km), 200 µg ml⁻¹; ampicillin (Ap), 200 µg ml⁻¹.

General methods. DNA manipulations were carried out according to standard procedures (Maniatis *et al.*, 1982). Transformation of *E. coli* or *S. marcescens* was done as described by Takagi & Kisumi (1985).

DNA sequencing. The nucleotide sequence of the plasmid containing the mutant-type *proBA* genes from *S. marcescens* SP187 was determined by dideoxy chain-termination method (Sanger *et al.*, 1977) with the aid of a sequencing system purchased from Takara Shuzo (Kyoto, Japan). After subcloning DNA fragments into pUC18, pUC19, pHSG298 and pHSG299, ordered deletion subclones were obtained by unidirectional digestion (Yanisch-Perron *et al.*, 1985). The DNA chains were labelled with [α -³²P]dCTP (400 Ci mmol⁻¹; 15 TBq mmol⁻¹). Nucleotide sequence data were analysed by using the computer program GENETYX (Software Development, Japan).

Proline excretion. To determine the mutation responsible for proline overproduction, we constructed four hybrid *proBA* plasmids containing mutations in the *proBA* operon. The 2.9 kb *Bam*HI-*Eco*RI *proBA* fragments of pWP142 and pY1720 were subcloned into the corresponding sites of pLG339 to give pWPK342 (wild-type) and pYIK920 (mutant), respectively. Plasmid pWPK100, carrying the *Ppro2* mutation, was constructed by replacing the 0.4 kb *Nco*I-*Ava*I fragment containing the promoter region of pWPK342 with one isolated from pYIK920 (see Fig. 1). Plasmid pWPK010, carrying the *proB1* mutation, was obtained by replacing the 0.9 kb *Ava*I-*Sph*I fragment containing the *proB* gene of pWPK342 with one isolated from pYIK920 (see Fig. 1). *S. marcescens* TT392 was used as a host for these plasmids.

Proline excretion was determined by an auxanographic cross-feeding test using a proline auxotroph, *S. marcescens* SP139 (Sugiura *et al.*, 1985). A minimal agar plate seeded with this strain at 5×10^8 cells ml⁻¹ was spotted with cells of the strain to be tested and incubated at 30 °C for 24–48 h. Excretion was scored positive if a halo of satellite growth of the indicator cells was observed.

Assay of enzyme activity. Cells were grown at 30 °C in 500 ml flasks containing 200 ml minimal medium. For the assay of GK activity the cells were harvested in the exponential growth phase, washed with 50 mM-Tris/HCl (pH 7.2) containing 1 mM-dithiothreitol and disrupted by sonication. The cell extracts were desalted with a Pharmacia PD-10 column and used for hydroxamate assay of GK activity as described by Hayzer & Leisinger (1980). The standard assay, containing 50 mM-L-glutamate, was used. The difference between values with and without the addition of 1 M-L-proline to the reaction mixture represented the proline-inhibitable GK activity. The proline-inhibitable GK of *S. marcescens* TT392 containing pWPK010 was inhibited by 1 M-L-proline to the level of the same strain without the plasmid.

For the assay of β -galactosidase, cells were harvested in the exponential growth phase, washed with saline, and resuspended in 100 mM-sodium phosphate buffer (pH 7.0) containing 10 mM-KCl, 1 mM-MgSO₄ and 50 mM-2-mercaptoethanol. After disruption by

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype*	Source or reference
<i>E. coli</i> K12 JM109	$\Delta(lac-proBA)$ <i>thi</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>S. marcescens</i> Sr41 TT392	Nuc ⁻ r ⁻ Ap ^s Km ^s	Takagi & Kisumi (1985)
SP103	<i>putA</i>	Sugiura <i>et al.</i> (1985)
SP139	<i>putA proB/A</i>	Sugiura <i>et al.</i> (1985)
Plasmids		
pBR322	Ap ^r Tc ^r	Bolivar <i>et al.</i> (1977)
pLG339	Km ^r Tc ^r	Stocker <i>et al.</i> (1982); derived from pSC101
pUC18 } pUC19 }	Ap ^r <i>lacZ</i> α	Yanisch-Perron <i>et al.</i> (1985)
pHSG298 } pHSG299 }	Km ^r <i>lacZ</i> α	Takeshita <i>et al.</i> (1987)
pLGlacZ1	pLG339::lacZ	Omori <i>et al.</i> (1991)
pY1333	pKP1154::Ppro2 <i>proB1A</i> ⁺	Takagi <i>et al.</i> (1985)
pY1720	pBR322::Ppro2 <i>proB1A</i> ⁺	This study
pWP142	pBR322::proB ⁺ A ⁺	Omori <i>et al.</i> (1991)
pWPK342	pLG339::proB ⁺ A ⁺	This study
pYIK920	pLG339::Ppro2 <i>proB1A</i> ⁺	This study
pWPK100	pLG339::Ppro2 <i>proB</i> ⁺ A ⁺	This study
pWPK010	pLG339::proB1A ⁺	This study
pWPL10	pLGlacZ1::Ppro ⁺ <i>proB</i> ⁺	Omori <i>et al.</i> (1991)
pYIL10	pLGlacZ1::Ppro2 <i>proB</i> ⁺	This study

* Symbols used for relevant genotypes and phenotypes are as follows: Nuc⁻, no production of extracellular nuclease; r⁻, defect of a host restriction enzyme; *putA*, destruction of proline oxidase; *proB/A*, proline auxotrophy; *Ppro*, promoter of the *proBA* genes; *Ppro2*, up-mutation in the promoter enhancing transcription of the proline operon, located in the spacer region; *proB1*, lack of feedback inhibition of GK.

sonication, the resultant cell extracts were used for the assay of β -galactosidase. β -Galactosidase activities were measured using o-nitrophenyl β -D-galactoside as a substrate (Miller, 1972).

The units of GK and β -galactosidase activities are nmol product formed min⁻¹ (mg protein)⁻¹. Protein concentration was determined by the use of a Bio-Rad protein assay kit with bovine serum albumin as the standard.

Analysis of *proB-lacZ* expression. The transcriptional activity of the *proBA* promoter was measured by using the plasmid pLGlacZ1 (Omori *et al.*, 1991). We constructed a gene fusion by ligating the beginning of the *lacZ* structural gene in-frame to the region containing the *proBA* promoter and the 5' terminus of the *proB* gene. The *proB-lacZ* gene fusion plasmid, pYIL10, was constructed by cloning the 0.3 kb *Bam*HI-*Sac*II fragment of pY1720 into the filled-in *Bam*HI site of pLGlacZ1 (see Fig. 4). The proline-oxidase-deficient mutant of *S. marcescens*, SP103, was used as a host to prevent the degradation of L-proline added to the medium (Sugiura *et al.*, 1985).

In vitro transcription-translation analysis of plasmid DNAs. The prokaryotic cell-free coupled transcription-translation system from Amersham was employed for labelling proteins encoded by plasmids.

Fig. 1. Restriction maps of the *proBA* plasmids pYI333 and pYI720, and the base substitutions found in the mutant *proBA* operon. The restriction sites indicated on the map are defined. Open arrows show the ORFs and the promoter region (*P*) of the *proBA* operon. The positions of two base substitutions, *Ppro2* and *proB1*, and the resultant amino acid substitution in the *proBA* operon, are indicated.

Fig. 2. Base substitution found in the promoter region of the *proBA* operon. The nucleotide sequence of the promoter region of the mutant is shown. The distance in base pairs from the first transcription start site, which is designated +1, is given above the sequence. Potential $-10'$ and $-35'$ sequences are underlined. A region of dyad symmetry, centred at position 107-108 ($-34-35$), is shown by inverted arrows. The base change from a C to a T at nucleotide 127 (-16) is indicated by an arrow.

Plasmid DNA (0.4 μ g), prepared by CsCl density-gradient centrifugation, was added to the reaction mixture (5 μ l) containing L-[35 S]-methionine, according to the manufacturer's protocol. SDS-PAGE of 35 S-labelled proteins was carried out as described by Laemmli (1970). Labelled polypeptide bands were visualized by autoradiography.

Sensitivities to osmotic stress and to proline analogues. Osmotolerance and proline analogue resistance were measured as described by Sugiyama *et al.* (1985). To determine the sensitivity to osmotic stress, we used a *putA*⁺ strain, *S. marcescens* TT392, as host. Cells harbouring pWPK342, pWPK100 or pWPK010 were grown at 30 °C in the minimal medium containing 0.5% glucose as carbon source with or without the addition of 0.6 M-NaCl to increase osmolarity. To examine proline analogue sensitivity, the *putA* strain *S. marcescens* SP103 was used, to prevent the degradation of L-proline and proline analogues. To determine the sensitivities to DHP and TAC, cells carrying the above plasmids were grown in the minimal medium containing 0.5% glucose or 0.5% sodium succinate, respectively. Growth was estimated turbidimetrically at 660 nm with a Hitachi electric photometer (EPO-B type).

Results

Base substitutions in the mutant *proBA* operon

The mutant *proBA* operon from strain SP187 had been previously cloned into pKP1154, resulting in plasmid pYI333 (Takagi *et al.*, 1985). The 2.9 kb *NcoI*-*MluI* fragment of this plasmid was subcloned in the *Bam*HI-*Eco*RI interval of pBR322, creating pYI720, and was confirmed to contain the entire *proBA* genes by a complementation test. The structure of pYI720 carrying the mutant *proBA* operon was shown to be identical by restriction analysis to that of pWPK142 carrying the wild-type *proBA* (Omori *et al.*, 1991). The complete nucleotide sequence of the insert carried by pYI720 was determined. Subsequently, this insert was confirmed to

Fig. 3. Hybrid plasmid analysis of the mutations responsible for proline overproduction. *S. marcescens* TT392 was used as the host for plasmids. Proline excretion was detected by the presence of a halo of satellite growth of the indicator, *proB/A* strain SP139, surrounding the colony. The regions derived from the wild-type and mutant-type *proBA* operons are indicated by open and filled boxes, respectively. The promoter region of the *proBA* operon is indicated by *P*. The relative positions of amino acid substitutions are shown by open triangles.

include the two open reading frames coding for GK and GPR, a promoter region, and a terminator region. Nucleotide sequence comparisons with the wild-type *proBA* operon revealed two base substitutions in the mutant-type operon (Fig. 1). One, designated *Ppro2*, was a G-C to A-T transversion at nucleotide 127, in the spacer between the '–35' and '–10' sequences (Fig. 2). The other, designated *proBI*, was a G-C to A-T transversion at nucleotide 531 in the *proB* structural gene. This substitution predicts an amino acid substitution from an alanine to a valine at amino acid residue 117 in the mutant GK. Although the proline-overproducing mutation in the *pro* operon of strain SP187 was previously designated *dpr-1* (Sugiura & Kisumi, 1985a), here we have found that this mutation consists of two base substitutions in the operon. In this paper, we use *Ppro2* and *proBI* as genotypes of the mutations.

Effects of the individual base substitutions on proline production

We subcloned the wild-type and the mutant *proBA* fragments into a low-copy-number vector, pLG339, for stable expression of the *proBA* operon, generating pWPK342 and pYIK920, respectively. To elucidate the

Fig. 4. Constitution of *proB-lacZ* fusion plasmids and level of β -galactosidase activity in *S. marcescens* SP103 (*putA*). Restriction sites used for plasmid construction are indicated. Cells were grown in the minimal medium containing glucose as described in Methods.

Table 2. Resistance to osmotic stress and to proline analogues of *S. marcescens* harbouring different plasmids

The host strain was *S. marcescens* SP103, grown in minimal medium with the additions shown.

Plasmid	Generation time (h)							
	C source glucose					C source succinate		
	Control	0.6 M-NaCl	0.6 M-NaCl + 1 mM-L-Pro	1 mM-DHP	1 mM-DHP + 1 mM-L-Pro	Control	10 mM-TAC	10 mM-TAC + 1 mM-L-Pro
pWPK342	1.1	>10	2.6	>10	1.2	1.7	>10	3.6
pWPK100	1.1	>10	2.5	>10	1.0	1.8	>10	3.5
pWPK010	1.6	2.5	2.3	1.6	1.5	2.9	>10	3.2

Fig. 5. Analysis of polypeptides encoded by plasmids carrying the wild-type and mutant *proBA* genes. Plasmid-specified polypeptides were produced in S-30 cell-free extract from *E. coli*. The ^{35}S -labelled polypeptides synthesized *in vitro* were analysed by SDS-PAGE (12.5%, w/v, acrylamide) and visualized by autoradiography. Lane 1, no plasmid; lane 2, pBR322; lane 3, pWPK142; lane 4, pYI720. The migration of protein standards is indicated on the left. *bla* indicates β -lactamase encoded by the plasmids used.

effects of each mutation on proline production, two *proBA* plasmids, pWPK100 and pWPK010, containing either the *Ppro2* or the *proB1* mutation, respectively, were constructed. The mutant and the wild-type *proBA* plasmids were introduced into cells of *S. marcescens* TT392 (wild-type) and tested for proline excretion (Fig. 3). Plasmids pWPK010 and pYIK920, carrying the *proB1* mutation, exhibited proline excretion, while cells containing the *proB*⁺ plasmids, pWPK342 (wild-type) and pWPK100 (*Ppro2*), did not excrete proline. These results indicated that the *proB1* mutation, a base substitution in the *proB* gene, which predicts an amino acid substitution from an alanine to a valine at amino acid residue 117 in the mutant-type GK, causes the proline overproduction. No difference in proline excretion was observed between pWPK010, carrying the *proB1* mutation, and pYIK920, carrying both *Ppro2* and *proB1* mutations.

Lack of feedback inhibition of GK encoded by the *proB1* gene

We have previously reported that the wild-type GK produced by the plasmid carrying the wild-type *proBA* operon is very sensitive to proline-mediated feedback inhibition (Omori *et al.*, 1991). The activity of GK produced by the *proB1* plasmid, pWPK010, was examined for feedback inhibition. The concentration of L-proline causing 50% inhibition was 11 mM for the mutant GK and 0.016 mM for the wild-type (results not shown).

Elevated expression of the *proBA* operon caused by the *Ppro2* mutation

To examine the effect of the *Ppro2* mutation on gene expression, we constructed the *proB-lacZ* gene fusion plasmid pYIL10, carrying the *Ppro2* mutation, as described for pWPL10 by Omori *et al.* (1991). β -Galactosidase activities were examined using cells harbouring each of the two gene fusion plasmids (Fig. 4). The promoter carrying the *Ppro2* mutation showed about fourfold greater transcriptional activity than the wild-type promoter. SDS-PAGE analysis of *in vitro* protein synthesis confirmed the overproduction of the GPR and GK proteins, of 44 and 39 kDa, respectively, directed by the plasmid pYI720, carrying the *Ppro2* mutation (Fig. 5).

The role of the two regulatory mutations in osmotolerance and in proline analogue resistance

We determined the sensitivities to osmotic stress and to proline analogues of *S. marcescens* SP103 harbouring the *Ppro2* and *proB1* plasmids (Table 2). Cells carrying the plasmid with the wild-type *proB*⁺ *A*⁺ operon, pWPK342, showed high sensitivities to an increased osmolarity and to the proline analogue DHP. The *Ppro2* plasmid, pWPK100, was similar to the wild-type plasmid in the sensitivities it conferred to osmolarity and DHP. Although the *proB1* plasmid, pWPK010, impaired the growth rate of its host in media of normal osmolarity, it conferred an enhanced tolerance to osmotic stress and a high resistance to DHP. However, this plasmid did not give TAC resistance to its host.

Cells harbouring pYIK920, which carries both *Ppro2* and *proB1* mutations, grew more slowly than those harbouring the *proB1* plasmid, pWPK010. Plasmid pYIK920 was unstable in *S. marcescens* and cells carrying this plasmid segregated mutants that no longer excreted proline. Plasmids isolated from these mutants could complement the *proBA* mutation. Nucleotide sequence analysis of these plasmid DNAs revealed a

Fig. 6. Comparison of the amino acid sequence of *S. marcescens* GK with that of *E. coli* GK. The partial amino acid sequences of *S. marcescens* wild-type GK (SM; Omori *et al.*, 1991) and *E. coli* GK (EC; Deutch *et al.* 1984; Csonka *et al.*, 1988) are presented in the one-letter code. Identical residues are indicated by vertical lines. The amino acid substitutions found in *E. coli* *proB74* (Csonka *et al.*, 1988; Dandekar & Uratsu, 1988) and DHP^R (Deutch *et al.*, 1984; Rushlow *et al.*, 1984) mutants are shown by arrows below the sequence. The amino acid substitution found in *S. marcescens* *proB1* (this work) is indicated above the sequence.

base substitution at nucleotide 531 that predicted a change of the amino acid 117 from a valine (mutant) to an alanine (wild-type) in the GK protein.

Discussion

Our results indicate that *S. marcescens* SP187, a proline-hyperproducing mutant, possesses two mutations in the *proBA* operon. The proline overproduction and the resultant resistance to both osmotic stress and a proline analogue are due to one of these mutations, *proB1*. This mutation is a single base substitution in the *proB* gene which directs an amino acid substitution of the GK protein. The GK enzyme specified by this mutant-type *proB* gene is 700-fold less sensitive to proline-mediated feedback inhibition than the wild-type. Another mutation, *Ppro2*, is a base substitution located in the spacer region of the promoter (Hawley & McClure, 1983; Harley & Reynolds, 1987).

The *Ppro2* mutation stimulates expression of *proBA* fourfold over that of the wild-type. Expression of *proBA* is unlikely to be subject to any transcriptional regulation (Omori *et al.*, 1991). Hence, enhanced *proBA* expression caused by the *Ppro2* mutation is not likely to be due to a loss of repression but rather to the up-mutation of the promoter. Up-mutations in the spacer region have been reported in the *lac* (Dickson *et al.*, 1975) and *gal* (Busby *et al.*, 1984) promoters of *E. coli*. Stefano & Gralla (1982) have proposed that the spacer region is responsible for the local conformation of the '−10' and '−35' regions which make contact with RNA polymerase. The *Ppro2* mutation may cause a subtle change in the conformation of the DNA of the promoter region. Although the *Ppro2* mutation alone confers neither proline excretion nor resistance to osmotic stress and proline analogues, it results in the overproduction of the GK and GPR proteins, and consequently enhances proline production. This indicates that in *S. marcescens*, the regulation of proline synthesis depends mainly on the feedback inhibition of GK and that the *proB1* mutation which

alters the allosteric properties of GK is a key mutation leading to proline overproduction. Transductional analysis of strain SP187 showed that the two mutations in the *proBA* region were acquired at the step of selecting DHP-resistant mutant SP105, the grandparent of strain SP187. Strain SP105 was selected as the best proline producer among DHP-resistant mutants (Sugiura & Kisumi, 1985a).

Nucleotide sequence analysis of the *proBA* genes of mutants segregated from cells carrying the mutant *proBA* plasmids revealed that the *proBA* genes carry a reverse mutation in *proB*, a change of amino acid 117 from a valine (mutant) to an alanine (wild-type) in the GK protein. This mutation is the cause of the loss of proline productivity reported by Takagi *et al.* (1985).

S. marcescens is similar to *E. coli* in the structure of the *proBA* operon (Omori *et al.*, 1991). The GK proteins produced by both bacteria consist of 367 amino acid residues and homology between them is 88%. In *E. coli*, *proB* mutants resistant to a proline analogue and overproducing proline have been isolated (Csonka, 1981; Mahan & Csonka, 1983; Smith *et al.*, 1984). The *proB* mutations, DHP^R and *proB74*, result in a loss of allosteric inhibition of GK and depend on single amino acid substitutions: at amino acid residues 143 and 107 in the DHP^R GK and *proB74* GK, respectively (Csonka *et al.*, 1988; Dandekar & Uratsu, 1988; Rushlow *et al.*, 1984). The *S. marcescens proB* mutation reported here, designated *proB1*, predicts an amino acid substitution at amino acid residue 117 in the GK protein. These three amino acid substitutions are all located in a region (amino acid residues 107–143) which lies apart from the sequence (amino acid residues 160–190) homologous to aspartokinase (Omori *et al.*, 1991) (Fig. 6). The *S. marcescens proB1* and *E. coli proB74* mutations confer osmotolerance on the cells, but the DHP^R mutation does not. This region of GK is probably important for binding of proline to the allosteric site of the wild-type GK. Further kinetic studies of the mutant GKs, analysis of the other DHP resistance mutations, and X-ray crystallographic analysis of mutant-type GKs, should clarify this.

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